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REVIEW ARTICLE

Factors affecting somatic embryogenesis in conifers

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Abstract: This review seeks to examine the extreme response of isolated somatic plant cells of apical meristematic tissues of mature conifer trees towards specific stress conditions *in vitro* resulting in somatic embryogenesis. Signal molecules regulating embryo development have been described in angiosperms, but very little is known about somatic rejuvenation in conifers. Recent studies on cloning of mature conifers provide new perspectives on signal molecules on cellular dedifferentiation into the embryogenic pathway. Our recent studies show that signal molecules such as butenolide, calcium ions, salicylic acid, antioxidants, amino acids, triacontanol and 24-epibrassinolide all play an important role in the conversion of somatic cells into an embryogenic pathway in many recalcitrant pines. This constitutes a major breakthrough in forest biotechnology with many practical applications in clonal forestry.

Key words: cloning; commercial forestry; *in vitro*; micropropagation; thin cell layers

Abbreviations: AGP, arabinogalactan protein; ASA, acetyl salicylic acid; BR, brassinosteroid; DCR, Durzan and Gupta medium; DTT, dithiothreitol; GlcN, *N*-glucosamine; EGTA, ethylene glycol-bis-(β-aminoethyl ether)- N,N,N',N'-tetraacetic acid; GlcNAc, *N*-acetylglucosamine; La^{3+}, lanthanum chloride; LCO, lipophilic chitin oligosaccharide; LM, Litvay medium; OG, oligogalacturonide; MS, Murashige and Skoog medium; MSG, Becwar medium; Nod, nodulation; PEM, pro-embryogenic mass; PGR, plant growth regulator; SA, salicylic acid; SSW, smoke-saturated water; TDZ, thidiazuron; tTCL, transverse thin cell layer; TRIA, triacontanol; *WUS*, homeobox transcription factor WUSCHEL

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Introduction

Somatic embryogenesis: general considerations

Somatic or body cells have the capability to reinitiate an entire ontogenic program, termed somatic embryogenesis (Feher et al. 2003; Malabadi et al. 2011). The totipotency of somatic plant cells is a specific and scientifically exciting phenomenon, which is based on the developmental program of plants (Ikeda-Iwai et al. 2003; Namasivayam 2007; Malabadi et al. 2009a). Therefore, the differentiation of somatic cells is reversible (Feher et al. 2003). It can be best demonstrated in an *in vitro* system where somatic plant cells can regain their totipotency and are capable of forming embryos through the developmental pathway of somatic embryogenesis (Feher et al. 2003; Malabadi et al. 2009a). This is due to the presence of specific undifferentiated organ cells, the meristems. The activity of meristematic cells is maintained, initiated or stopped by endogenous as well as environmental signals (Charles and Fletcher 2003; Feher et al. 2003). Environmental and endogenous factors together determine the developmental fate of plants through the activation or inactivation of meristems (Feher et al. 2003; Feher 2006). Plant cells attempt to establish a new programme through changes in pH gradients of all cell compartments, arresting differentiated functions, reactivating the cell cycle and re-organising gene expression as well as metabolism (Feher et al. 2003).

Under *in vitro* conditions, one or a few somatic cells of the plant or explant have to be competent to receive a signal (endogenous or exogenous). This then triggers the pathway of embryogenic development (commitment) leading to somatic embryo formation (Feher et al. 2003). For a particular genotype or plant, the *in vitro* forms of somatic embryogenesis, the optimum conditions (potential, competence, induction, and commitment) have to be experimentally optimized (Feher et al. 2003). Although *in vitro* somatic embryogenesis is practiced in many tissue culture laboratories throughout the world using many conifer species, genotypes and explants, the biological background of the process is still largely unknown and not well studied. Therefore, we still do not know how and why competence or commitment is

achieved by a somatic cell or what is the real trigger (signal) initiating embryo development. It was presumed earlier that the potential of somatic embryogenesis is determined at the level of the genotype, which is clearly proved by the successful transfer of the embryogenic capacity between embryogenic and recalcitrant genotypes *via* sexual crossing (Feher et al. 2003). Recalcitrance could be resolved by optimizing *in vitro* growth conditions of plants or by proper explant selection. Genetic determinants therefore, may only serve to define the conditions when and where embryogenic competence can be expressed (Feher 2006). Thus, embryogenic potential is largely defined by the developmental programme of the plant as well as by environmental cues or stimuli (Feher 2006). Unlike carrot or alfalfa, where somatic embryogenesis can develop on all organs of seedlings, in conifers, embryogenic competence is restricted to certain tissues of a given genotype. Tissue culture studies support the view that there exists a kind of gradient in the embryogenic response among various plant organs (Feher et al. 2003; Feher 2006). The embryogenic potential is highest in tissues with embryonic origin and decreases in other explants of the same plant species. However, even if embryogenic competence seems to be lost in somatic plant cells, it can potentially be regained (Feher et al. 2003; Feher 2006). In this indirect somatic embryogenesis pathway, an intermediate callus formation phase is required in order to express embryogenic potential (Feher 2006). Therefore, differentiated plant cells do not lose their developmental potential during normal development but retain plasticity, that is, they are capable of dedifferentiating and acquiring new developmental fates (Feher et al. 2003), the so-called universal totipotency of plant cells.

Somatic embryogenesis in woody plants and conifers

All woody plants exhibit a phase change. Mature trees can not be regenerated under normal standard *in vitro* conditions and alternative approaches include the application of an abiotic stress (temperature, chemical, osmotic) to alter phase change (von Aderkas and Bonga 2000; Karami and Saidi 2009). Stress can have a rejuvenating effect on plant tissues (Poethig 1990; von Aderkas and Bonga 2000). At present, embryogenic systems derived from vegetative shoot apices or secondary needles of mature pines have been well established in at least a few conifers (Bonga and Pond 1991; Ruaud et al. 1992; Bonga and von Aderkas 1993; Ruaud 1993; Westcott 1994; Smith 1994, 1997, 1999; Paques and Bercetche 1998; Bonga 1996, 2004; Malabadi et al. 2002, 2004; Malabadi and van Staden 2003, 2005a, 2005b, 2005c, 2006; Malabadi 2006; Malabadi and Nataraja 2006a, 2006b; Aronen et al. 2007, 2008; Malabadi and Nataraja 2007a, 2007c 2007f, 2007g; Malabadi et al. 2008a, 2008b, 2008c; Park et al. 2009; Malabadi and Teixeira da Silva 2011), and an embryogenic system could be used for genetic transformation studies. These are major breakthroughs in forest biotechnology, especially considering the time-scale of a conifer's life-cycle, that would certainly solve some of the current problems of tree breeding (Malabadi and Nataraja 2007e). Another important advantage of using vegetative shoot apices of mature pines as the starting material for somatic embryogenesis is that cells are actively dividing, hence their higher regeneration capacity, and serve as the best starting material for many experimental studies (Malabadi and Nataraja 2007e). After re-initiation of cell division, and a period of proliferation of the release of explant cells in the presence of signal molecules released due to environmental abiotic stress conditions, a small subset of the cell population becomes embryogenically competent. These competent cells are generally in the form of clusters of small cytoplasmic cells whose nuclei show active cell division. The application of techniques to follow the developmental fates of individual single competent cells present during cloning of mature conifers has revealed a striking heterogeneity in embryogenic competent cells and early cell division patterns that were nonetheless not readily apparent in the morphology of the resulting somatic embryos. In spite of major success in cloning of mature conifers, the role of signal molecules has not been well studied with respect to embryogenic competence of somatic cells in conifers. In this review, in celebration of 2011, the UN International Year of Forests, we attempt to summarize the involvement of signal molecules during induction phase of embryogenic competence of somatic cells derived from transverse thin cell layers (tTCLs) of apical meristematic tissue of mature conifers.

Signal molecules in plant somatic embryogenesis

The role of signal molecules in somatic embryogenesis has been reported in several plant species, but only in a few conifers, including Norway spruce (*Picea abies*). Several components in the conditioned growth medium promote somatic embryogenesis. These components include chitinases (Egertsdotter et al. 1993; Dyachok et al. 2002), and arabinogalactan proteins (AGPs; Egertsdotter and von Arnold 1995). Such signals are effective across genera: a sugar beet (*Beta vulgaris*) endochitinase can stimulate early development of *P. abies* somatic embryos (Egertsdotter and von Arnold 1995, 1998). A basic chitinase secreted by *Pinus caribbaea* embryogenic tissue, but not by non-embryogenic tissue, acts upon AGP from embryogenic tissue but not AGPs from non-embryogenic tissue (Dyachok et al. 2002; Cairney and Pullman 2007). AGPs, glycosylated polypeptides consisting of up to 90% carbohydrate, are widely distributed, are commonly found in tissue culture, and are capable of stimulating somatic embryogenesis when added to a weakly embryogenic cell line (Dyachok et al. 2002; Cairney and Pullman 2007). Oligosaccharides released from AGPs by a chitinase act as signal molecules stimulating somatic embryogenesis while chitinase-modified AGPs are extracellular molecules capable of controlling or maintaining the embryogenic competent cell state (van Hengel et al. 2001; Dyachok et al. 2002). The relationship between AGPs, chitinases and lipophilic chitin oligosaccharides (LCOs) and their manner of stimulating embryogenesis has been well documented (van Hengel et al. 2001; Dyachok et al. 2002; Wiweger 2003; Feher et al. 2003). AGPs isolated from immature seeds had an increased capacity to promote embryogenesis when

pretreated with chitinase EP3 or AGP3 were extracted from endosperm (van Hengel et al. 2001; Dyachok et al. 2002). Therefore, AGP-mediated control of embryogenesis may be regulated by differential gene expression, and/or differential processing of the AGP-polypeptide moiety. AGPs and LCO as well as chitinases (EP3 and CH4) can stimulate somatic embryogenesis in Norway spruce (Daychok et al. 2002). It has been suggested that LCOs are parts of AGPs that are released by chitinases: van Hengel and coworkers (2001) showed that LCO-like molecules are released from AGPs after they are hydrolysed by chitinases. Dyachok et al. (2002) reported that the endogenous LCO acts as a signal molecule stimulating pro-embryogenic masses (PEMs), and early embryo development in Norway spruce. Embryogenic cultures of Norway spruce are composed of PEMs and somatic embryos of various developmental stages. Auxin is important for PEM formation and proliferation (Dyachok et al. 2002). Depletion of auxin blocks PEM development and causes large-scale cell death in *P. abies* (von Arnold et al. 2002). Extracts of the media conditioned by embryogenic cultures stimulate development of PEM aggregates in auxin-deficient cultures (Dyachok et al. 2002). Partial characterization of the conditioning factor shows that it is a lipophilic, low-molecular-weight molecule, which is sensitive to chitinase and contains GlcNAc (oligosaccharide backbone β-1,4-linked *N*-acetylglucosamine) residues (Dyachok et al. 2002). On the basis of this information, Dyachok et al. (2002) proposed that the factor is an LCO. The amount of LCO correlates to the developmental stages of PEMs and embryos, with the highest level in the media conditioned by developmentally blocked cultures. LCO, however, is not present in non-embryogenic cultures of *P. abies* (Dyachok et al. 2002; Wiweger 2003). Cell death, induced by withdrawal of auxin, is suppressed by an extra supply of endogenous LCO or nodulation (Nod) factor from *Rhizobium* sp. NGR234. The effect can be mimicked by a chitotetraose or chitinase from *Streptomyces griseus* (Dyachok et al. 2002; Wiweger 2003). Nod factors are produced by bacteria belonging to the genera *Rhizobium*, *Azorhizobium* and *Bradyrhizobium* in response to plant flavonoids (Spaink 1996; Dyachok et al. 2002; Wiweger 2003). Different rhizobia produce different sets of Nod factors with specific modifications, and these appear to determine host specificity (Staehelin et al. 1994b; Dyachok et al. 2002; Wiweger 2003). However, Nod factors uniformly consist of an oligosaccharide backbone of GlcNAc tri-, tetra- or pentasaccharide, with an N-linked fatty acid moiety replacing the N-acetyl group on the non-reducing end (Wiweger 2003). The length of the oligosaccharide chain, the acetylation at the non-reducing end and the sulfation at the reducing end of the LCO, influence the stability of the molecule against degradation by chitinases (Staehelin et al. 1994; Wiweger 2003). Nod factors are known to induce cell divisions in the root cortex of the host legume, leading to the formation of nodules (Schultze and Kondorosi 1996; Spaink 1996; Wiweger 2003). Rhizobial LCOs and chitin oligosaccharides stimulate the earliest stages of nodulation probably by perturbing the auxin flow in the root, and this auxin transport inhibition is probably mediated by endogenous flavonoids (Wiweger 2003). In spruce, Nod factors influence embryo development while oligogalacturonide (OG) induces changes in the developmental pattern of somatic embryos but this is strictly dependent on the developmental stage of the treated embryos (Wiweger 2003). Treatment of embryos at the globular stage resulted in the inhibition of the elongation of the axis and the formation of a multiple shoot apex while treatment of embryos at early stages of development results in severe abnormalities during later development (Wiweger 2003).

Therefore, these results confirmed that endogenous LCO acts as a signal molecule stimulating PEM and early embryo development in Norway spruce (Dyachok et al. 2002; Wiweger 2003). It has long been known that Nod factors produced by rhizobia induce cell divisions in the root cortex of the host legume, leading to the formation of nodules (Wiweger 2003). Furthermore, in Norway spruce, Nod factors can substitute for auxin and cytokinin to promote cell division (Dyachok et al. 2002; Wiweger 2003). In embryogenic maize cells, the extracellular matrix surface network has also been shown to contain arabinogalactan proteins (AGPs), as well as the antibody JIM4 arabinogalactan protein epitope, that were not present on the surface of non-embryogenic cells (Wiweger 2003; Namasivayam et al. 2010).

Plant growth regulators (PGRs) play important roles as signal molecules and regulators of growth and development in plants. An endogenous auxin pulse is one of the first signals leading to the induction of somatic embryogenesis (Wiweger 2003). Auxin and cytokinins are the main PGRs in plants involved in the regulation of cell division and differentiation. The influence of exogenously applied auxins, particularly 2,4-dichlorophenoxy acetic acid (2,4-D), on the induction of somatic embryogenesis using vegetative shoot buds and secondary needles of mature pines, embryo cloning and mature zygotic embryos are well documented (Malabadi et al. 2004; Malabadi and van Staden 2005a, 2005b, 2005c, 2006; Malabadi 2006; Malabadi and Nataraja 2006a, 2006b; Aronen et al. 2007; Malabadi and Nataraja 2007f, 2007h; Malabadi et al. 2008a, 2008b, 2008c). Endogenous PGR levels, however, can be considered as major factors in determining the specificity of cellular responses to these rather general stress stimuli. In addition to the absolute requirement of exogenous auxins for sustained growth in *in vitro* cultures, plant cells may produce substantial amounts of the native auxin, indole-3-acetic acid (IAA) (Feher et al. 2003). Higher endogenous IAA concentrations are associated with increased embryogenic response in various plant species. Among the different auxin analogues used to induce somatic embryogenesis, 2,4-D is by far the most efficient and, therefore, this synthetic PGR is used in the majority of embryogenic cell and tissue culture systems (Feher et al. 2003). 2,4-D above a certain concentration has a dual effect in these cultures, as an auxin (directly or through endogenous IAA metabolism) and as a stressor (Feher et al. 2003). 2,4-D is an auxinic herbicide with diverse effects associated with its phytotoxic activity, which can not be ascribed simply to an auxin overdose (Feher et al. 2003). Auxinic herbicides have been shown to interact with ethylene and abscisic acid (ABA) synthesis, increasing the cellular levels of these called stress hormones (Feher et al. 2003). During cloning of mature pine trees, a combination of α-naphthalene acetic acid (NAA), 2,4-D and 6-benzyl

adenine (BA) at particular concentrations induced embryogenic tissue on DCR (Gupta and Durzan 1985) induction medium. The optimum concentration the external PGRs was 22.62 μM 2,4-D, 26.85 μM NAA and 8.87 μM BA (Malabadi et al. 2004), and transverse thin cell layers (tTCLs), the ideal explants, induced embryogenic tissue in many pines such as *Pinus kesiya*, *P. roxburghii*, *P. wallichiana*, *P. patula*, *P. sylvestris*, *P. pinea* and *P. pinaster* (Malabadi et al. 2004; Malabadi and van Staden 2005a, 2005b, 2005c, 2006; Malabadi 2006; Malabadi and Nataraja 2006a, 2006b; Aronen et al. 2007; Malabadi and Nataraja 2007f, 2007h; Malabadi et al. 2008a, 2008b, 2008c). Higher or lower concentrations of NAA, 2,4-D and BA resulted in the induction of non-embryogenic tissue. Interestingly, cell cultures of different ages had different sensitivities to cytokinin treatment. In pines, the formation of somatic embryos is influenced by the application of ABA in the maturation medium, without PGRs (Malabadi and van Staden 2003, 2005a, 2005b, 2005c). This finding suggests that, as a stress signal, exogenous ABA is effective only in the absence of external PGRs (Feher et al. 2003). Therefore, the dedifferentiation of a somatic cell is influenced by many factors such as stress conditions, and in such a case, simultaneous activation of auxin and stress responses may be key events in cellular adaptation, causing genetic, metabolic and physiological reprogramming, which results in the embryogenic competence (totipotency) of somatic plant cells (Feher et al. 2003).

Role of signal molecules in cloning of mature conifers

In vitro somatic embryogenesis is associated with artificial conditions, high levels of exogenous PGRs and many other stress factors. These extreme and stressful conditions may result in a general stress response in cells showing activity of signaling molecules involved in the conversion of somatic cells isolated from apical meristematic tissues (i.e., young tissue) of mature conifers (Feher et al. 2003; Feher 2006; Karami and Saidi 2009). A large number of cellular events that have to be coordinated during the induction of embryogenic tissue define together only a narrow window that indeed permits the initiation and progression of embryogenic development (Feher et al. 2003). Therefore, not all cells of an explant subjected to the same treatment are capable of developing into somatic embryos, explaining why various explants, genotypes and species need different conditions for successful induction of embryogenic tissue in conifers.

In the following sections the most important signaling molecules that play an important role in the conversion of somatic cells isolated from the shoot apical meristem of many recalcitrant mature conifers are described.

(Ca^{2+})-mediated signaling

Calcium ions (Ca^{2+}) function as a key regulator of many cellular and physiological events in plants; Ca^{2+} functions as a second messenger in the signal transduction of a variety of environmental stimuli, which regulate and mediate plant embryogenesis and also as a key regulator of many cellular and physiological events (Poovaiah and Reddy 1993). Current models of Ca^{2+}-mediated signalling emphasize the significance of a transient change, usually an increase, in cytoplasmic calcium concentration, followed by the perception of such changes by calcium-binding proteins. The involvement of Ca^{2+} in a wide variety of stimulus-response pathways in plant cells raises several questions concerning how the same messenger can regulate different responses (Feher et al. 2003). The amplitude, duration, frequency and location of the Ca^{2+} signal can be considered as key features in the determination of different messages (Feher et al. 2003). The environmental stimuli and signaling events that trigger and regulate plant embryogenesis are largely unknown. The dependence of somatic embryogenesis on external and internal calcium concentrations has been demonstrated in different plant systems, including conifers. The elevated Ca^{2+} concentration counteracts the inhibitory effect of 2,4-D on embryo development (Feher et al. 2003). In carrot, somatic embryogenesis activated calmodulin (CaM) localized to regions undergoing rapid cell division, and an increase in the level of CaM mRNA was observed during globular and heart-shaped stages (Feher et al. 2003). Transfer of cells of the PEM onto PGR-free medium to promote somatic embryo development resulted in a several-fold increase in the uptake of exogenous Ca^{2+} as well as the Ca^{2+} level in the symplast. This could be blocked by chelating exogenous Ca^{2+}, which arrested somatic embryo development, but still allowed cell proliferation to continue (Feher et al. 2003). Blocking Ca^{2+}-mediated signalling resulted in an 85% decrease in somatic embryo formation frequency. This response indicated that CaM or Ca-dependent protein kinase could be involved in this process (Anil and Rao, 2000; Feher et al. 2003). Although there are data showing that CaM levels are increased in dividing cells, there were no significant changes detected in CaM levels (Feher et al. 2003) or CaM methylation and CaM-binding protein levels during carrot, sandalwood and alfalfa somatic embryo induction. However, an unknown Ca-binding protein was reported to be transiently induced in the early phase of somatic embryo induction in an alfalfa cell culture (Feher et al. 2003).

Experiments with calcium-channel blockers and calcium chelators used in somatic embryogenesis of sandalwood (*Santalam album*) and carrot indicated that the influx of exogenous calcium is essential for the initiation of somatic embryogenesis (Anil and Rao 2000; Malabadi and van Staden 2006). These observations suggest an intermediary role for Ca^{2+} during plant somatic embryogenesis. Increasing evidence has demonstrated that the elevation of cytoplasmic-free calcium ($(Ca^{2+})^{cyt}$) plays an important role in the response of plants to cold pretreatment shock (Malabadi and van Staden 2006). Such elevation of $(Ca^{2+})^{cyt}$ has been proposed to mediate a variety of physiological and developmental processes occurring at low temperature. A regulatory role involving changes in $(Ca^{2+})^{cyt}$ during somatic embryogenesis has been reported for sandalwood (Anil and Rao 2000; Malabadi and van Staden 2006). Increased exogenous calcium concentration favors somatic embryo formation in some species. Therefore, one can infer that cold-enhanced somatic embryogenesis could be strongly

related to cold-induced $(Ca^{2+})^{cyt}$ elevation by the influx of exogenous Ca^{2+} during cold pretreatment (Malabadi and van Staden 2006). In an embryogenic carrot cell suspension, an upward shift in the exogenous Ca^{2+} concentration (from 10^{-3} to 10^{-2} M) at the time of transfer to auxin-free embryo differentiation medium increased the number of somatic embryos approximately two-fold (Jansen et al. 1990; Feher et al. 2003).

To validate the role of exogenous Ca^{2+} as a second messenger in the induction or regulation of embryogenesis, a study was conducted in *Pinus patula* that investigated the necessity of a Ca^{2+} pool for embryogenesis (Malabadi and van Staden 2006). During this study exogenous Ca^{2+} influenced somatic embryogenesis using vegetative shoot apices of mature trees in three genotypes. The percentage of somatic embryogenesis in *P. patula* was significantly affected by increasing the exogenous Ca^{2+} concentration from 0 to 4 mM on the cold pre-treatment medium (I) and during the establishment of embryogenic suspension cultures. The highest percentage of explants showing embryogenesis (PP3: 8%, PP13: 10%, PP18: 3.5%; PP3 = *P. patula* no. 3; PP13 = *P. patula* no. 13; PP18 = *P. patula* no. 18) was recorded for three *P. patula* genotypes after the addition of 4 mM of exogenous Ca^{2+} during the cold pretreatment or during cell suspension culture when compared to controls (0 mM Ca^{2+}) (PP3: 4.5%, PP13: 3.7%, PP18: 2.0%). The incorporation of exogenous Ca^{2+} at 2 mM during cold pretreatment at 2°C and during the establishment of embryogenic cell suspension cultures did not have a marked effect on embryogenesis in *P. patula* but an increase in the exogenous Ca^{2+} concentration to 5 mM significantly reduced the percentage of somatic embryogenesis following cold pretreatment and during establishment of cell suspension cultures. Therefore, 4 mM of exogenous Ca^{2+} was the optimum concentration for embryogenesis in *P. patula*. Hence, exogenous Ca^{2+} at 4 mM in DCR basal medium during cold pretreatment supported the expression of cold-enhanced capacity for somatic embryogenesis in *P. patula*. Furthermore, when the residual Ca^{2+} in the cold pretreatment medium was chelated with 2 mM ethylene glycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), somatic embryogenesis in *P. patula* was significantly inhibited in pretreated suspension cultures compared with the control. These results suggest that cold-enhanced embryogenesis was related to Ca^{2+} in the medium used for cold pretreatment and that an optimum concentration (4 mM) was required during cold pretreatment for the expression of cold-enhanced embryogenesis in *P. patula* (Malabadi and van Staden 2006). Transfer of cells of a proembryonic cell mass onto PGR-free medium to promote somatic embryo development resulted in a several-fold increase in the uptake of exogenous Ca^{2+} as well as the Ca^{2+} level in the symplast (Feher et al. 2003). This could be blocked by chelating exogenous Ca^{2+} ions, which arrested somatic embryo development, but still allowed cell proliferation to continue. This response indicates that CaM- or Ca^{2+}-dependent protein kinase could be involved in this process (Anil and Rao 2000; Feher et al. 2003). Additional data support that Ca^{2+}-dependent protein kinases (CDPKs) are involved in the signaling pathways during the formation of somatic embryos in sandal wood (Anil and Rao 2000).

To further investigate whether the incorporation of exogenous Ca^{2+} in DCR medium during cold pretreatment plays a role in cold-enhanced somatic embryogenesis, lanthanum chloride ($LaCl_3$ or La^{3+}) and EGTA at 2 and 5 mM were added to the cold pretreatment medium to examine their effects on subsequent somatic embryogenesis in *P. patula* (Malabadi and van Staden 2006). However, suspension cultures pre-incubated in DCR medium containing either $LaCl_3$ or EGTA for two weeks displayed no growth when compared to the control. $LaCl_3$ and EGTA at 2 and 5 mM completely negated the cold-enhanced capacity for embryogenesis in *P. patula*. Furthermore, $LaCl_3$ and EGTA at 5 mM greatly inhibited somatic embryogenesis, suggesting that the influx of exogenous Ca^{2+} during cold pretreatment was also required for the expression of cold-enhanced somatic embryogenesis. The arrest of embryogenesis with Ca^{2+}-chelated culture conditions (DCR basal medium with $LaCl_3$ and EGTA at 2 or 5 mM) suggests that exogenous Ca^{2+} is indeed required for somatic embryogenesis. Concomitantly, the inhibition of somatic embryogenesis by plasma membrane Ca^{2+} channel blockers confirmed the need of a Ca^{2+} pool for the process of embryogenesis. Culture treatments with Ca^{2+} antagonists further confirmed the need for an exogenous Ca^{2+} pool for embryogenesis (Malabadi and van Staden 2006).

It has been suggested that exogenous Ca^{2+} ions play a role as a second messenger during sandalwood somatic embryogenesis (Anil and Rao 2000). The chelation of exogenous Ca^{2+} with EGTA or the initiation of exogenous Ca^{2+} movement across membranes with the Ca^{2+} channel blockers $LaCl_3$, nifedipine, verapamil and bepridil reduced the frequency of embryogenesis in sandalwood (Anil and Rao 2000; Feher et al. 2003). The results reported by Malabadi and van Staden (2006) also showed that incubation of suspension cells in the medium containing $LaCl_3$ resulted in the loss of the cold-enhanced capacity for somatic embryogenesis in *P. patula* suggesting that the influx of exogenous Ca^{2+} during cold pretreatment may be essential for cold-enhanced somatic embryogenesis. Although a preliminary experiment showed that somatic embryogenesis in $LaCl_3$-treated cultures could recover after La^{3+} was removed by washing, cold treatment of 5 mM $LaCl_3$ for two weeks resulted in a significant inhibition of somatic embryogenesis.

Antioxidants and amino acids

Explant browning and its subsequent death, generally attributed to phenolic compounds, is a major unsolved problem in the initiation of tissue cultures, especially of woody plants. Phenolic reactions, frequently manifested as darkening of explants, can lead to their death (Malabadi and van Staden 2005b). Free radicals are frequently generated by wounding and may result in an increase in the activity of peroxidase and catalase enzymes, which act to overcome the effect of oxidizing radicals (Benson 2000; Malabadi and van Staden 2005b). Therefore, reducing contact with atmospheric oxygen reduces the rate of oxidation of phenolics at wound sites. This resulted in the technique of submersion of explants in water as a means to reduce tissue oxidation (Malabadi and van Staden 2005b). Several antioxidants like cysteine, dithiothreitol and a combination of ascorbic acid (AA) with citric acid (CA) have been evaluated, with varying success, in the management of phenolic damage to excised tissue. Pre-

treatment of thin shoot apical dome sections or tTCLs of *Pinus patula* with different concentrations of antioxidants (CA, AA, cysteine, dithiothreitol (DTT) (0, 0.01, 0.05, 0.1%), CA/AA (0.01, 0.1, 0.5%) and tri-potassium citrate (KC) combined with CA in equal concentrations (0.01 0.1, 0.05% KC/CA), for 10 min, or including antioxidants directly in the DCR culture media, reduced somatic embryo production compared with controls pretreated with sterile distilled water only (Malabadi and van Staden 2005b). However, pretreatment of explants (tTCLs) with the antioxidants had a positive effect by reducing the degree of tissue necrosis and reducing the oxidation of phenolic compounds. The explants thus remained green without browning. This resulted in the best survival of explants used for the initiation of embryogenic cultures. However, the percentage of surviving cultures and somatic embryo production decreased when pretreated with antioxidants such as CA, AA, AA/CA and KC/CA respectively. In all three genotypes of *P. patula*, the untreated controls showed a higher percentage of embryogenic culture initiation than when pretreated with antioxidants. The exception being DTT at 0.1% which increased the percentage of embryogenic cultures and degree of somatic embryo production. Higher concentrations of DTT decreased the percentage of embryogenic cultures and somatic embryo production during cloning of mature trees of *P. patula* (Malabadi and van Staden 2005b).

The addition of almost all antioxidants to DCR culture media or a pretreatment reduced production of somatic embryos during cloning of mature *P. patula* trees (Malabadi and van Staden 2005b). Antioxidants generally function as part of a system where the accepted electrons are transferred to another molecule and release the primary acceptor to again function as an electron acceptor; they may also act as electron donors under certain circumstances, enhancing the oxidation process (Benson 2000). The prevention of oxidation during tissue excision for culture is important and prevention of direct contact of the tissues with air is frequently more advantageous than using chemical mediators such as antioxidants that may interfere with subsequent developmental events (Malabadi and van Staden 2005b). In that study, DTT had a positive effect on the initiation of embryogenic cultures at 0.1%. Presently it is not known how DTT influences embryogenesis. This simple procedure of preparing explant material using DTT (0.1%) prior to culture results in a reduction of tissue death caused by browning (phenolic oxidation), consequently enhancing initiation of embryogenesis of shoot apical domes of mature *P. patula* trees (Malabadi and van Staden 2005b) and could thus solve the problem of browning in other conifer somatic embryogenesis protocols.

Somatic embryo production can be promoted by the addition of amino acids. Amino acids, when used during embryo maturation and somatic seedling recovery in three *P. patula* genotypes (PP3, PP13, and PP18), resulted in a favorable recovery of somatic embryos (Malabadi and van Staden 2005b). In PP3, lower concentrations (1.0, 2.0, 5.0 mM) of a standard amino acid solution mixture of 17 amino acids (L-glutamine (1.0 $g \cdot l^{-1}$), alanine (0.8 $g \cdot l^{-1}$), proline (1.0 $g \cdot l^{-1}$), lysine (2.0 $g \cdot l^{-1}$), glycine (0.5 $g \cdot l^{-1}$), L-arginine (2.5 $g \cdot l^{-1}$), leucine (2.0 $g \cdot l^{-1}$), phenylalanine (0.82 $g \cdot l^{-1}$), serine (1.05 $g \cdot l^{-1}$), isoleucine (1.3 $g \cdot l^{-1}$), valine (2.3 $g \cdot l^{-1}$), histidine (0.4 $g \cdot l^{-1}$), threonine (1.2 $g \cdot l^{-1}$), tyrosine (0.6 $g \cdot l^{-1}$), asparagine (1.5 $g \cdot l^{-1}$), tryptophan (0.6 $g \cdot l^{-1}$), and cystine (0.2 $g \cdot l^{1}$)), did not have a significant effect on somatic embryogenesis (Malabadi and van Staden 2005b). On the other hand, a higher concentration (20.0 mM) decreased the percentage of somatic embryogenesis and somatic seedling recovery in *P. patula* (Malabadi and van Staden 2005b). The highest percentage of somatic embryogenesis (19%) and somatic seedling recovery (15) was with genotype PP3 and was recorded with 10.0 mM of the amino acid mixture (Malabadi and van Staden 2005b). Garin et al. (2000) reported that a combination of amino acids incorporated in the maturation medium either had no effect or decreased somatic embryo production of *Pinus strobus*, depending upon the embryogenic line. However, in *Picea glauca*, somatic embryos matured in the absence of glutamine, but in the presence of casein hydrolysate and inorganic nitrogen. Smith (1994) supplemented with amino acids in the maturation medium of *Pinus radiata* to improve somatic embryo production and plantlet recovery, with a solution of seven amino acids and reported significant effects on somatic embryogenesis (Malabadi and van Staden 2005b). In other plant species, such as alfalfa and *Picea glauca* some amino acids had either a negative or no effect on somatic embryo maturation. During cloning of mature trees of *P. patula*, apart from dithiothreitol at 0.1%, pre-treatment of tTCL explants or incorporation of antioxidants in the DCR basal nutrient medium had a negative effect on the initiation of embryogenic cultures, somatic embryo production and plantlet recovery.

Salicylic acid

The PGR salicylic acid (SA), when applied to plants, affects diverse physiological processes, such as plant defense responses to pathogens and abiotic stress, as well as plant growth and development (Tuteja et al. 2010). Exogenous SA and acetylsalicylic acid (ASA) can also enhance somatic embryogenesis in *Pinus roxburghii* (Malabadi et al. 2008a, 2008b).

Therefore, the current approach of cloning mature conifer trees using SA has many practical applications, particularly in clonal forestry, with the possibility of solving many problems related to conifer somatic embryogenesis (Malabadi et al. 2008a, 2008b). SA is a mobile molecule, which is capable of acting as a cell signal that senses, amplifies, and transmits information from a cell and might help in programming towards embryogenesis during cloning. Secondly, SA is involved (together with nitrogen oxide, hydrogen peroxide, and other metabolites) in the function of several signal systems, unifying them into an intricate network of regulatory interactions. Perhaps embryo differentiation may share some of the intermediates in the salicylate signal pathway (Tuteja et al. 2010).

In a study of mature cloned *P. roxburghii* trees, pre-treatment (5 min) of shoot-tip tTCL explants of 10 different Chir pine genotypes with different concentrations of SA did not induce embryogenic tissue any more than the control (Malabadi et al. 2008a, 2008b). All genotypes showed a mixed response in embryogenic tissue induction following pretreatment of tTCLs with

SA. The pre-treatment of tTCLs from any of the 10 genotypes with 0.1, 0.2 and 0.4 mg^{-1} SA could not effectively increase the percentage of somatic embryogenesis when compared to the control. Two genotypes of *P. roxburghii*, PR-05 and PR-92, failed to induce embryogenic tissue following pre-treatment of explants with any concentration of SA, i.e., these two genotypes were completely recalcitrant to this treatment. Pretreatment of explants with higher concentrations (2.0–5.0 mg^{-1}) of SA might have had a toxic effect, which resulted in the browning of explants without callus formation in all 10 genotypes of *P. roxburghii*. The percentage of responsive explants that could induce embryogenic tissue increased (significantly in some cases) from 7% to 12% in PR-811, 3% to 5% in PR-32, 6% to 8% in PR-805, and 11% to 16% in PR-821 following the pre-treatment of 1.0 mg^{-1} SA when compared with the control in *P. roxburghii*. This trend was also similar with 0.5 mg^{-1} SA where the percentage of responsive based explants for inducing embryogenic tissue increased from 7% to 9% in PR-811 and 11% to 14% in PR-821, respectively. Therefore, pre-treatment with 0.5 or 1.0 mg^{-1} SA was optimum at least in a few *P. roxburghii* genotypes (PR-811, PR-32, PR-805, PR-821) for improving the percentage of somatic embryogenesis. Incorporation of 1.0 mg^{-1} SA in the induction medium was optimum for all 10 genotypes by increasing the percentage of somatic embryogenesis compared to the control. The highest percentage (31%) of somatic embryogenesis was recorded in PR-821 and PR-46. For PR-05, in particular, the addition of 1.0 mg^{-1} SA to the induction medium was very beneficial since in the control this genotype failed to induce somatic embryogenesis. This clearly indicates the positive role of SA as a signaling molecule during cloning of mature *P. roxburghii* trees. In these studies, SA alone (i.e. without PGRs) did not induce somatic embryogenesis and resulted in the browning of explants and callus. Microscopic observation showed simple, elongated parenchymatous cells without any sign of cleavage polyembryony. SA, when combined with 22.6 μM 2,4-D, 26.8 μM NAA and 8.9 μM BA in the induction medium improved the percentage of somatic embryogenesis in *P. roxburghii*. However, in PR-05, this synergistic mix induced only 3% somatic embryogenesis while in PR-05 it failed to induce somatic embryogenesis. Hence, the combination of SA with other PGRs such as 2,4-D/NAA/BA might be beneficial in inducing somatic embryogenesis in Chir pine (Malabadi et al. 2008a 2008b). Ethylene inhibits differentiation in plants, although, during a study on the cloning of mature *P. roxburghii* trees, SA is suspected to have promoted embryo development by inhibiting ethylene biosynthesis (Malabadi et al. 2008a, 2008b). Another hypothesis is that SA increases the activity of superoxide dismutase (Rao et al. 1997), and inhibits the activities of ascorbate peroxidase and catalases, thus leading to endogenous H_2O_2 accumulation (Rao et al. 1997). The cloning of mature *P. roxburghii* trees was successful following the addition of 1.0 mg^{-1} SA in DCR induction medium in most *P. roxburghii* genotypes tested (Malabadi et al. 2008a, 2008b). On the contrary, the external application of SA to tTCL explants prior to cloning was not effective for inducing somatic embryogenesis. Therefore, SA was shown to play an important role in inducing somatic embryognesis in conifers, showing a positive sign of conversion of a somatic cell towards embryogenic pathway (Malabadi et al. 2008a, 2008b).

TRIA-mediated signaling

The plant growth stimulating property of triacontanol (TRIA) was already reported in woody plants (Tantos et al. 2001). TRIA, a long 30-carbon primary alcohol, is a naturally occurring plant growth promoter (Ries and Houtz 1983) and a component of plant epicutcular waxes (Azam et al. 1997). TRIA induced somatic embryogenesis in *Pinus roxburghii* and *P. kesiya*, the first reports for conifers (Malabadi and Nataraja 2005, 2007c). TRIA had a high potential to induce embryogenic tissue using mature zygotic embryos in three genotypes of *P. roxburghii.* During that study, mature zygotic embryos cultured on full-strength LM (Litvay et al. 1985) basal medium supplemented with 9.0 μM 2,4-D and lower concentrations of TRIA (4, 5 and 7 $\mu g \cdot 1^{-1}$) induced white mucilaginous embryogenic callus in all three genotypes. Microscopic observation of callus revealed actively dividing elongated cells with few undergoing cleavage polyembryony. Mature zygotic embryos induced a higher percentage of white glossy non-embryogenic callus with lower (1, 2 $\mu g \cdot 1^{-1}$) and higher concentrations of TRIA (15, 20, 25, 30 $\mu g \cdot 1^{-1}$) on full-strength LM basal medium in all three genotypes. The most effective range of TRIA which induced white-mucilaginous embryogenic callus on full-strength LM basal medium containing 9.0 μM 2,4-D was 4-7 $\mu g \cdot 1^{-1}$ in all three genotypes. The highest percentage of white mucilaginous embryogenic callus was induced on full-strength LM basal medium supplemented with 9.0 μM 2,4-D and 7 $\mu g \cdot 1^{-1}$ TRIA (initiation medium I) (Malabadi and Nataraja 2007c). The percentage of initiation of embryogenic cultures was not similar in all the three genotypes. In genotype PR481, 67.4% of embryogenic cultures formed while the highest percentage of embryogenic cultures (87.5%) was initiated in PR810. Lowest percentages of embryogenic cultures (40.8%) were initiated in genotype PR35. Therefore, 9.0 μM 2,4-D and 7 $\mu g \cdot 1^{-1}$ TRIA supplemented in full-strength LM basal medium is the optimum for the initiation of *P. roxburghii* embryogenic cultures (Malabadi and Nataraja 2007c). A lower concentration of TRIA may be biologically effective because of the sensitivity of whole explants to extremely low doses of TRIA (Biernbaum et al. 1998). TRIA, or a metabolite of TRIA, a secondary messenger, moves rapidly in plants after initial application and influences enzymes related to carbohydrates metabolism in plants and growth processes (Ries and Houtz 1983). TRIA-induced embryogenesis improved both initiation of embryogenic cultures and the recovery of plantlets in *P. kesiya* and *P. roxburghii* (Malabadi and Nataraja 2005, 2007c). Therefore, TRIA can be effectively used as a growth promoter in conifer somatic embryogenesis for the improvement of existing protocols to induce higher percentage of embryogenic cultures. In another recent study, an efficient somatic embryogenesis protocol was developed for the first time using TRIA as a PGR in *Catharanthus roseus* (Malabadi et al. 2009b). In that study, friable embryogenic callus was induced from thin sections of shoot tips collected from field-grown plants on MS medium

supplemented with 2.0 μM 2, 4-D and 5.0 μM TRIA (induction medium I). Subculture of embryogenic callus on maturation medium supplemented with 5.0 μM ABA without any other PGRs induced cotyledonary stage somatic embryos. The highest percentage of somatic embryogenesis (85.0%) was recorded in genotype III, with a total of 14 somatic seedlings recovered per gram fresh weight of embryogenic tissue. Somatic embryos were successfully germinated on half strength MS medium without PGRs. TRIA clearly acts as a signaling molecule in inducing somatic embryogenesis in conifers.

Smoke-saturated water

Smoke is an important factor involved in fire and post-fire germination cues (van Staden et al. 2000; Jain et al. 2008). Farmers have traditionally used fire and smoke in grain drying practices (van Staden et al. 2000) and smoke from the combustion of plant material stimulates seed germination in a wide range of species (Light and van Staden 2004). A highly active germination promoting compound has recently been identified as a water-soluble butenolide, 3-methyl-2*H*-furo [2, 3-c] pyran-2-one, from the smoke of burnt fynbos *Passerina vulgaris* Thoday and the grass *Themeda triandra* L. (van Staden et al. 2004) as well as from the combustion of cellulose (Flematti et al. 2004). The recent identification of the germination cue, butenolide from smoke will now allow for research into the physiological action of smoke on seed germination. Smoke-saturated water (SSW) does not have any significant effect on the germination period of somatic embryos in all the three genotypes of *P. wallichiana* although it did affect the total number of somatic embryos that germinated (Malabadi and Nataraja 2007a; Malabadi et al. 2009), and even though it promoted vandal orchid growth (Malabadi et al. 2008c). In another study, the effect of butenolide, 3-methyl-2*H*-furo [2, 3-c] pyran-2-one was tested for its effect on somatic embryogenesis with an important species for commercial horticulture, *Baloskion tetraphyllum* (Restionaceae) (Ma et al. 2006). It was observed that when somatic embryos of *B. tetraphyllum* were transferred to basal medium (MS) supplemented with 0.067 μM butenolide, the development of growth-competent somatic embryos was enhanced using different explants such as shoots and coleoptiles (Ma et al. 2006). Butenolide resulted in a high frequency of somatic embryos progressing to plantlets, and a higher number of plantlets per explant compared to non-butenolide (control) media for both shoot and coleoptile explants in *B. tetraphyllum* (Ma et al. 2006). These observations suggest that the active ingredient(s) in SSW play a regulatory role in plant development. The number of somatic embryos doubled following the addition of SSW at either the explant or induction stage compared to the untreated control in *P. wallichiana* (Malabadi and Nataraja 2007a; Malabadi et al. 2009c). The inductive signals for the initiation of somatic embryogenesis of *P. wallichiana* were BA, NAA and 2,4-D. SSW without BA, NAA or 2,4-D did not induce any form of cell proliferation; however, SSW appeared to act synergistically with the inductive signal (Malabadi and Nataraja 2007a; Malabadi et al. 2009c). Collectively taken, these observations suggest that SSW acts like a PGR rather than a nutritional additive. Smoke may have an action similar to cytokinins in breaking seed dormancy, and by modulating the sensitivity of the tissue to PGRs, activation of enzymes or by modifying the receptor molecules (Jain et al. 2008). Therefore, SSW has a stimulatory role during cloning of mature trees of *P. wallichiana*, and thus stimulates the conversion of somatic cell into an embryogenic pathway (Malabadi and Nataraja 2007a; Malabadi et al. 2009c).

24-Epibrassinolide-mediated signaling

Brassinosteroids (BRs) are a group of naturally occurring steroidal lactones that include brassinolide and its analogs. In several bioassays, they have been reported to affect cell elongation, division and differentiation of plant cells (Vardhini et al. 2010). Embryogenic tissue could be initiated in conifers on media using 24-epibrassinolide (24-epiBR) (Pullman et al. 2003). In various bioassays, brassinolide has been shown to be more active than, or synergistic with, auxins such as IAA or NAA (Brosa 1999). Ronsch et al. (1993) reported an improvement in the rooting efficiency and survival of Norway spruce seedlings using 22S, 23S-homobrassinolide. Hu et al. (2000) suggested that 24-epiBR may promote cell division through CycD3, a D-type plant cyclin gene through which cytokinin activates cell division: 24-epiBR can substitute cytokinin in the culture of callus and suspension cells. However, very few reports are available on the effect of BRs in plant micropropagation and tissue culture.

More recently, in conifers, mature zygotic embryos produced white mucilaginous embryogenic tissue on MSG (Becwar et al. 1990) medium containing 9.0 μM 2,4-D and 24-epiBR at 0.5, 1.0 and 2.0 μM (Malabadi and Nataraja 2007d). Mature zygotic embryos produced the highest percentage of embryogenic tissue on half-strength MSG medium supplemented with 9.0 μM 2,4-D and 2.0 μM 24-epiBR (initiation medium) in all three genotypes of *Pinus wallichiana* tested. The highest percentage of somatic embryogenesis (91.5±3.0a) was recorded in genotype PW106. On the other hand, the lowest percentage of somatic embryogenesis (60.8±4.0 b) was obtained in genotype PW21 (Malabadi and Nataraja 2007d). Although, little information is available for conifers, BRs have been isolated from conifers (Kim et al. 1990) and exogenous applications of BRs to pine seedlings and spruce cuttings have shown improved root growth, whole plant growth, or both (Ronsch et al. 1993; Rajasekaran and Blake 1998). Pullman et al. (2003) reported that use of brassinolide at 0.1 μM improved the percentage of embryogenic cultures in loblolly pine (*Pinus taeda*), Douglas fir (*Pseudotsuga menziesii*), and Norway spruce. They also showed that brassinolide increased the weight of loblolly pine embryogenic tissue by 66% and stimulated initiation of embryogenic tissue in the more recalcitrant families of loblolly pine and Douglas fir, thus compensating somewhat for genotypic differences in initiation (Pullman et al. 2003). BRs are of ubiquitous occurrence in plants and elicit a wide spectrum of physiological responses (Vardhini et al. 2010). In angiosperms, BRs have several effects, including stimulating cell division, ethylene production, and adventitious tissue formation and increasing resistance to abiotic stress (Brosa 1999). These results

indicated ample evidence that BRs possess a broad spectrum of biological activities compared to the known plant PGRs, including gibberellin, auxin and cytokinin-like activities (Vardhini et al. 2010).

Genetic cues, activation and signaling in somatic embryogenesis

The processes during which somatic cells acquire embryogenic competence obviously involve the re-programming of gene expression patterns (Chugh and Khurana 2002; Namasivayam 2007). Cell differentiation depends on the proper and sequential expression of key genes required for morphogenesis. Reprogramming of cells during cloning confirmed that the morphology, physiology and metabolism of the cells are significantly altered due to dedifferentiation, activation of cell division and a change in cell fate (Chugh and Khurana 2002; Namasivayam 2007). Most of the results of gene expression are derived from the three best-studied systems: carrot, alfalfa and chicory (Namasivayam 2007). There is only one gene known to play a role in the acquisition of embryogenic competence in plant cells. This is the somatic embryogenesis receptor kinase (SERK) gene isolated by Schmidt et al. (1997). In carrot, SERK expression was shown to be characteristic of embryogenic cell cultures and somatic embryos, but its expression ceased after the globular stage (Schmidt et al. 1997; Namasivayam 2007). It could also be detected in zygotic embryos up to the early globular stage, but not in unpollinated flowers or in any other tissue. Using the SERK promoter fused to the luciferase gene and video cell tracking, it was also shown that SERK-expressing single cells could develop into somatic embryos (Schmidt et al. 1997; Chugh and Khurana 2002; Namasivayam 2007). In contrast to carrot and *Arabidopsis*, SERK expression in *Dactylis glomerata* was maintained beyond the globular stages of embryogenesis in meristematic regions (Namasivayam 2007). Based on all of these experiments, the expression of the SERK gene may be used as a marker of embryogenic competence. And the SERK may represent a possible candidate for mediating signals which are required to initiate the embryogenic development (Feher et al. 2003). Other leucine rich repeat-containing receptor-like kinases are known to be involved in different developmental processes in plants, such as the ARABIDOPSIS CLAVATA and ERECTA proteins (for review, see Fletcher and Meyerowitz 2000) and the *Petunia* PRK1 protein, which is involved in signaling during pollen development and pollination (Mu et al. 1994; Feher et al. 2003). Another family of transcription factors, those containing the so-called MADS-box domain, are also important regulators of many plant developmental processes (Jack 2001; Feher et al. 2003). The expression of promoter-GUS gene fusions is not restricted to embryogenesis and it is more likely linked to a juvenile tissue state.

During the last few years the homeobox transcription factor WUSCHEL (WUS) has been shown to cause dedifferentiation when expressed on somatic cells followed by a production of new stem cells that can lead to somatic embryogenesis or organogenesis (Zuo et al. 2002; Arroyo-Herrera et al. 2008). The homeobox gene *WUS* is required to specify stem cell identity. WUS was originally identified as a central regulator of shoot and floral meristems in *Arabidopsis* (Mayer et al. 1998) where it is expressed in a small group of cells, and is required to maintain the overlying stem cells undifferentiated (Arroyo-Herrera et al. 2008). In *Arabidopsis*, WUS has been found to be sufficient to ectopically induce stem cells (Zuo et al. 2002). Furthermore, WUS has been shown to be part of a WOX family of proteins. The WOX proteins maintain a common regulatory sequence between them (Arroyo-Herrera et al. 2008). The function still unknown in the majority of the WOX members however it is known that they are expresses asymmetrically and may be involved in the differentiation process (Arroyo-Herrera et al. 2008). Arroyo-Herrera et al. (2008) found that expression of WUS in coffee plants can induce calli formation as well as a 400% increase somatic embryo production. These results showed that transgenic expression of the transcription factor WUS can be useful to increase somatic embryogenesis in heterologous systems (Arroyo-Herrera et al. 2008). However, a critical developmental stage and additional hormonal requirements are required for the induction of embryogenesis by WUS in coffee (Arroyo-Herrera et al. 2008).

Considerable efforts have been made to identify genes with altered expression patterns during somatic embryogenesis. Most of these genes, however, are up regulated only in the late developmental stages, suggesting that they do not play a direct role in the vegetative-to-embryogenic transition (Arroyo-Herrera et al. 2008; Karami et al. 2009). However, very little is known about the molecular mechanism that mediates the vegetative-to-embryogenic transition. Therefore, it is necessary to employ a genetic approach to dissect the signal transduction pathway during somatic embryogenesis (Zuo et al. 2002). In a functional screen using a chemical-inducible activation-tagging system, Zuo et al. (2002) identified two alleles of *Arabidopsis* gene PGA6 who's induced over expression caused high-frequency somatic embryo formation in all tissues and organs tested, without any external plant hormones (Zuo et al. 2002). Upon inducer withdrawal, all these somatic embryos were able to germinate directly, without any further treatment, and to develop into fertile adult plants. PGA6 was found to be identical to WUS, a homeodomain protein previously shown to be involved in specifying stem cell fate in shoot and floral meristems (Zuo et al. 2002). Transgenic plants carrying an estradiol-inducible XVE-WUS transgene can copy the phenotype of pga6-1 and pga6-2. These results suggest that WUS/PGA6 also plays a key role during embryogenesis, presumably by promoting the vegetative-to-embryogenic transition and/or maintaining the identity of the embryonic stem cells (Zuo et al. 2002). The pga6 mutation-dependent cell-fate reprogramming can occur in the presence or absence of external plant hormones, although the local concentration of endogenous growth regulators might play an important role in the vegetative-to-embryogenic transition. In addition, the hormone-independent somatic embryogenesis in pga6 strikingly resembles zygotic embryo development. These observations suggest that PGA6 plays a critical regulatory role during embryogenesis, which is probably involved in maintaining embryonic cell identity (Zuo et al. 2002). Hence, observation of Zuo et al. (2002) confirmed that WUS is capable of promoting the vege-

tative-to-embryonic transition, and eventually somatic embryo formation, suggesting that the homeodomain protein also plays a critical role during embryogenesis, in addition to its function in meristem development (Zuo et al. 2002). Presumably the highly restrictive expression of WUS hallmarks the putative embryonic organizing centre which, in turn, may give rise to stem cells during embryogenesis and later development (Zuo et al. 2002).

In all these *in vitro* systems PGRs, particularly auxin or 2,4-D, are essential for the induction of somatic embryogenesis, although auxin has to be promptly removed from the medium for somatic embryos to form. This is because appropriate auxin transport and distribution are needed for embryo development and pattern formation (Zuo et al. 2002). WUS transient overexpression causes highly embryogenic callus formation in the presence of auxin, whereas it directly induces somatic embryo formation from different plant organs in the absence of any exogenous auxin. Therefore, it appears that WUS can reprogram cell fate, bypassing the auxin requirement or simply taking advantage of the endogenous auxin flux (Zuo et al. 2002). Moreover, no callus phase, or at least only few cell-division cycles, is sufficient to induce cells to restart a totally new embryogenic pathway in tissues of plants that over-express *WUS* (Zuo et al. 2002). However, there is at least one part of the plant that WUS can not reprogram to form embryos: the shoot apical meristem (Trigiano and Gray 2010). The presence or absence of some factors in the shoot apex could favour one (a shoot meristem organizer) or the other WUS function (an embryo organizer) (Zuo et al. 2002; Trigiano and Gray 2010). Finally, the finding that pga6 gain-of-function mutations or over expression of WUS result in somatic embryo formation from vegetative tissues at high frequency should have a significant impact on plant biotechnology, and provides a convenient and attractive model system for many aspects of plant biology research (Zuo et al. 2002). In another development, 12 well-conserved miRNAs and cleavage products of their target mRNAs in needle tissues of the conifer *Pinus resinosa* (red pine) (Cairney and Pullman 2007). The APETELA2 (AP2) gene is regulated by miR172 in *Arabidopsis* and the putative target site is conserved in the gymnosperms *Cycas revoluta*, *Ginkgo biloba* and *Gnetum parvifolium*, implying conservation of regulation over the course of revolution (Cairney and Pullman, 2007). Recently, several miRNAs involved in seed formation in *Pinus taeda* were identified and demonstrated their presence in both embryos and female gametophyte tissue (Cairney and Pullman 2007). MicroRNAs (miRNAs) are small noncoding endogenous RNA molecules approximately 20-24 nucleotides in length that down-regulate gene expression in a variety of eukaryotic organisms including poplar (*Populus trichocarpa*) (Cairney and Pullman 2007). However, certain miRNAs may be tree specific or exhibit tree-specific regulation: A number of miRNAs in *Populus trichocarpa* which show sequence conservation in *Arabidopsis* show different patterns of expression in *P. trichocarpa* and 10 experimentally validated poplar miRNAs are absent in *Arabidopsis* (Cairney and Pullman 2007).

Conclusion and future perspectives

Recently cloning of mature trees of conifers has been achieved successfully in many recalcitrant pines throughout the world. Signaling molecules and stress conditions (such as cold or heat or chemical stress) of the isolated somatic cells under given *in vitro* conditions will force the cells towards embryogenic pathway. Competent cells can respond to a variety of conditions by the initiation of embryogenic development. It can also be hypothesized that the initiation of somatic embryogenesis using mature conifers is a general response to a multitude of parallel signals (including growth regulators, stress factors, physiological status of buds, carbon source, calcium ions, and alteration of gene expression). Although genetic components clearly determine the potential of a species\genotypes to form somatic embryos, the expression of embryogenic competence at the cellular level is defined by developmental and physiological cues. Therefore, we conclude that both stress conditions and signaling molecules plays an important role in cloning of mature trees of conifers. Molecular analysis of signaling pathways and processes such as programmed cell death and embryo maturation indicates that many developmental pathways are conserved between angiosperms and gymnosperms. During the last few years the homebox transcription factor WUSCHEL (WUS) has been shown to cause dedifferentiation when expressed in somatic cells followed by the production of new stem cells that can lead to somatic embryogenesis. Further research could be required to define if over expression of WUS in recalcitrant pines can lead to somatic embryogenesis. Plants continuously maintain pools of totipotent stem cells in their apical meristems from which elaborate root and shoot systems are produced. The balance between the rate of cell division and differentiation is crucial for the ordered transition zones in the meristem. Recently for the first time, the expression of cDNA clones of genes involved in programming the apical meristem cells towards somatic embryogenic pathway influenced by external environmental stimulus like cold pretreatment has been reported in *P. roxburghii* (Malabadi and Nataraja 2007g). Differential display was used to isolate the genes which are expressed specifically in embryogenic tissue induced by cold-pretreatment of thin sections of vegetative shoot apices of mature trees of *Pinus roxburghii*. Of the 56 cold-enhanced embryogenic-associated cDNAs identified, 20 were cloned (Malabadi and Nataraja 2007g). During reverse northern hybridization, all the 20 clones selected generated a positive signal when probed with labeled cDNA from cold-enhanced embryogenic tissue, but no signal when probed with cDNA from the non-embryogenic tissue (control treatment). All the 20 clones thus contained inserts that were specific to cold-enhanced somatic embryogenesis. The identification of genes revealed the expression of *WUS* (unpublished data) in the embryogenic tissue derived from the apical meristematic tissue from mature trees of *P. roxburghii* (Malabadi and co-workers, unpublished work). This clearly indicates the involvement of *WUS* in the induction of embryogenic tissue from the apical meristem. Therefore, homebox transcription factor *WUS* might be involved in the mo-

lecular mechanism that mediates the vegetative-to-embryogenic transition during cloning of mature trees of *P. roxburghii* (Malabadi and co-workers, unpublished work). Therefore, it is very difficult to address the factors which are involved in the activation of stem cells of the apical meristematic tissue or activation of homebox gene WUS due to cold stress conditions leading to the conversation of somatic cells into embryogenic pathway in mature pines as was noticed in *Arabidopsis* and *Coffea canephora* (Arroyo-Herrera et al. 2008). At this point of time, we still not sure whether the cloning of mature trees of conifers is mainly due to stress-related differentiation or regulated by the expression of WUS-related conversion of somatic cells into an embryogenic pathway. A detailed study of identification of genes and stress related factors is still under way.

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